



Leaf gas exchange and isoprene emission in poplar in response to long-term experimental night-time warming and summer drought in a forest-steppe ecosystem[☆]

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ARTICLE INFO

Keywords:

Climate change

Photosynthesis

Isoprene synthase gene expression

Temperature

Biogenic volatile organic compounds

ABSTRACT

Climate change projections forecast an average warming in mean temperature and an associated change in the distribution and intensity of rainfalls. At the manipulation experiment in Kiskunság, Hungary, we aimed to study the effect of the soil drought and night-time warming conditions projected for the coming decades on leaf gas exchange, including photosynthetic rates and isoprene emission rates and on isoprene synthase expression levels of *Populus alba* sprouts (root suckers). During the years 2010 and 2011, warming treatment increased the air temperature 0.35 °C on average whereas drought treatment reduced the soil moisture by 13% on average in relation to control plots. The results highlighted important seasonal differences. Photosynthesis was limited by stomatal closure in summer and also isoprene emission and isoprene synthase expression levels were lower in summer than in autumn for all treatments. As a consequence, a negative relationship was found between temperature, ranging from 25 °C to 35 °C, and isoprene emission throughout all the study period and treatments. Results also showed significant treatments effects on isoprene parameters but only in July 2011. Isoprene emission decreased with drought, and isoprene synthase expression levels decreased with night-time warming. A positive relationship was found between isoprene emission and leaf internal carbon in drought treated poplar plants, which contrasted with our findings for control and warming treated plants. These results indicated either a change in the regulation of pyruvate distribution between isoprene synthesis and respiration under drought or a utilization of alternative carbon sources for isoprene emission.

1. Introduction

Isoprene (2-methyl-1,3-butadiene) is the most abundant biogenic volatile organic compound (BVOC), a reactive hydrocarbon that contributes to the production of tropospheric ozone in the presence of nitrogen oxides (NO_x) and sunlight (Hauglustaine et al., 2004) and to the formation and growth of aerosol particles in the atmosphere (Paasonen et al., 2013). It is emitted in large amounts by several tree species (Guenther et al., 1999; Kesselmeier and Staudt, 1999).

Isoprene is synthesized from dimethylallyl diphosphate (DMADP) through a reaction catalyzed by the enzyme isoprene synthase (ISPS)

(Schnitzler et al., 1996; Silver and Fall, 1995) and it is emitted through stomata to the atmosphere (Sharkey et al., 2001). Given the importance of BVOC emissions for the photochemistry of the atmosphere, there is great interest in determining how global change factors affect isoprene synthesis and emission. Moreover, *in planta*, isoprene is considered to be an important molecule for ameliorating abiotic stresses by preventing the accumulation of reactive oxygen species and protecting cell membranes against lipid peroxidation (Loreto and Schnitzler, 2010).

Synthesis and emission of isoprene are affected by several environmental factors, including photosynthetically active radiation (PAR) and temperature, which are the main input parameters of all

Abbreviations: A, photosynthesis; g_s, stomatal conductance; C_i, intercellular concentration of CO₂; ISPS, isoprene synthase; *PaISPS*, isoprene synthase from *Populus alba*; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; DOXP, 1,deoxy-D-xylulose-5 phosphate

[☆] This article is part of a special issue entitled “Experiments with trees: from seedlings to ecosystems” published at the journal Environmental and Experimental Botany 152C.

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<https://doi.org/10.1016/j.envexpbot.2018.04.005>

Received 25 July 2017; Received in revised form 9 April 2018; Accepted 11 April 2018

Available online 12 April 2018

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emission models (Guenther et al., 2006; Arneth et al., 2008; Monson et al., 2012). Therefore, the forecasted climate change scenario for the year 2100 (IPCC, 2013), with predicted changes in the distribution of rainfalls, more frequent summer droughts and a 1.5–3.7 °C increase in temperature, are highly likely to alter emissions of isoprene.

Night-time warming is expected to increase soil temperature, plant respiration, and potentially also nutrient cycling and daytime photosynthesis (Turnbull et al., 2002; Xia et al., 2009) and therefore may influence total BVOC emissions. Though Ibrahim et al. (2010) reported an increase in terpene emissions as a consequence of night-time warming other studies have shown no changes (Nogués et al., 2012) and therefore more research is needed to understand how changes in night-time temperature will alter the emissions.

The impact of drought on isoprene emission has also been studied, since drought is a major limitation for plant growth worldwide. Nevertheless, drought effect on isoprene emission is controversial since it is largely dependent on the stress severity. There are several studies showing reduction of isoprene emission rates under severe drought stress (Delfine et al., 2005; Peñuelas and Staudt, 2010) possibly as a consequence of reduced photosynthetic activity and reduced carbon availability. However, Brilli et al. (2007) demonstrated that, under these conditions, the observed residual emission of isoprene can be attributed to alternative carbon sources entering the DOXP/MEP pathway from sources other than photosynthesis (Lichtenthaler et al., 1997). Also, Centritto et al. (2011) found that the entire metabolism associated to isoprenoid synthesis in black poplar may be up or down-regulated under long periods of drought or high temperatures. On the other hand, isoprene inhibition rarely occurs when drought is mild (Peñuelas and Staudt, 2010).

ISPS gene expression has been reported not to vary during the first phases of drought and to decrease under severe drought stress (Fortunati et al., 2008). (Fortunati et al., 2008) also reported of decreases in ISPS activity before isoprene emission during drought development indicating that the main control of isoprene emission may take place at a transcriptional or post-transcriptional level under moderate drought stress.

This study focuses on the impacts of two field-manipulated environmental factors, namely night-time warming and more severe summer drought on isoprene emissions and isoprene synthase expression levels of white poplar (*Populus alba*). Though the effects of these changing climatic factors on the emission of BVOC have been already studied for different shrub and tree species, this is the first time that white poplar isoprene emission is assessed at ecosystem level *in situ* in a forest-steppe transitional zone and the first time that also isoprene synthase expression levels are determined under field conditions.

Given the existing uncertainties on this topic, the present study provides new data that helps us better understand: 1) the effect of long-term *in situ* manipulation of night-time warming and increased drought on isoprene emission and ISPS gene expression levels from *Populus alba*; 2) seasonal variability of isoprene emission and of the responses to manipulated warming and drought; 3) and help unravel the physiological mechanisms underlying the above-mentioned responses.

2. Material and methods

2.1. The experimental manipulation

The Hungarian experimental site Kiskunság is one of the seven experimental sites of the INCREASE EU-funded infrastructure project. This site is located in the Kiskunság National Park (46°53'N 19°23'E) (Kröel-Dulay et al., 2015). It represents a forest-steppe transitional biome between temperate deciduous forests and continental steppes in Europe. The dominant species are the deciduous clonal *Populus alba* and the perennial C3 bunchgrass *Festuca vaginata*. Other important species are the C3 grass *Stipa borysthonica* and the C4 grass *Cynodon dactylon*.

The coarse sand soil is very poor in organic matter (< 1%), and has

extreme heat and water regime, with soil temperature (in 5 cm depth) ranging from −17 °C to +43 °C and soil moisture content (V%/V%; 0–20 cm) ranging from 2% to 16% during 2010–2011.

The experimental site included nine plots (three replicates for each treatment, size 4 × 5 m) arranged in a random pattern. In three plots, air temperature was increased (0.4 °C increase in mean annual temperature) by covering the plots with a reflective material at night throughout the year (Kröel-Dulay et al., 2015). In three other plots, drought was simulated by covering the plots with a transparent waterproof material during rain events in May and June excluding 22% of the mean annual precipitation (Kröel-Dulay et al., 2015). Three untreated plots served as control. The experiment was built in 2001, and the treatments started in 2002. Drought was repeated in each summer (May and June), and warming treatment was running continuously.

Due to the transitional (grassland-shrubland-forest) character of Kiskunság, this ecosystem is expected to be sensitive to predicted changes in climate, thus being an ideal target system for climate change studies. Treatments and relative measurements (leaf gas exchange, isoprene emission rates and isoprene synthase expression levels) were carried out on *Populus alba* basal shoots, which are generally referred to as sprouts or suckers. The age of these poplar root suckers are not known, and can range from a few years to several decades. Their height ranged from 50 cm to 120 cm, and there were about 5–15 root suckers per plot (20 m²) (see picture in Supplementary material).

The technique used to manipulate the environmental conditions was characterized by a passive night-time warming technique and an automated covering system to make summer drought more severe (Beier et al., 2004). The night-time warming was induced throughout the year by covering vegetation and soil with aluminium curtains at night, simulating global warming. The curtains run along two rails placed on a light scaffolding 1.5 m tall. The warming plots were open during the day and automatically covered when light intensity at sunset was lower than 4 W m^{−2}. Severe summer drought was induced by covering the plots with transparent waterproof plastic curtains during rain events. A rain sensor activated a mechanism to cover plots with the curtains whenever it rained and remove them when the rain stopped. We continuously measured air temperature at 20 cm height (one sensor per plot) with a PT-100 resistance sensor-based thermometer. Soil moisture content (V/V%) was also continuously monitored in the 0–20 cm soil depth with a Campbell Scientific CS616 Water Content Reflectometer (one sensor per plot).

Three campaigns of measurements were carried out in total (Table 1). Mean air temperature and mean photosynthetic photon flux density (PPFD) in sampling dates are shown in Table 1. Air temperature and soil moisture values for each treatment throughout the study period are shown in Fig. 1. The temperature average increase by warming and the soil moisture reduction average by drought are shown in Table 2. The effects of warming on soil moisture and of drought on air temperature are not shown because they were negligible (Fig. 1). In the long term, the overall increase in Mean Annual Temperature in warming plots was 0.4 °C (Kröel-Dulay et al., 2015).

Rainfall during the years 2010 and 2011 was 1025 mm and 498 mm respectively. The 32% and the 25% of it was intercepted in years 2010 and 2011 respectively by the rain exclusion device in the drought treatment that was active during May and June. When considering only the spring season, drought curtains excluded the 69.79% and the

Table 1
Campaign dates and mean air temperature (°C) and mean maximum photosynthetic photon flux density (PPFD) in sampling dates.

Campaign dates	Temperature T (°C)	PPFD (μmol m ^{−2} s ^{−1})
192nd–194th 2010	29.00 ± 0.17	1992 ± 13.5
242nd–245th 2010	10.51 ± 0.05	1133 ± 17.9
189th–193rd 2011	27.13 ± 0.12	1685 ± 64.8

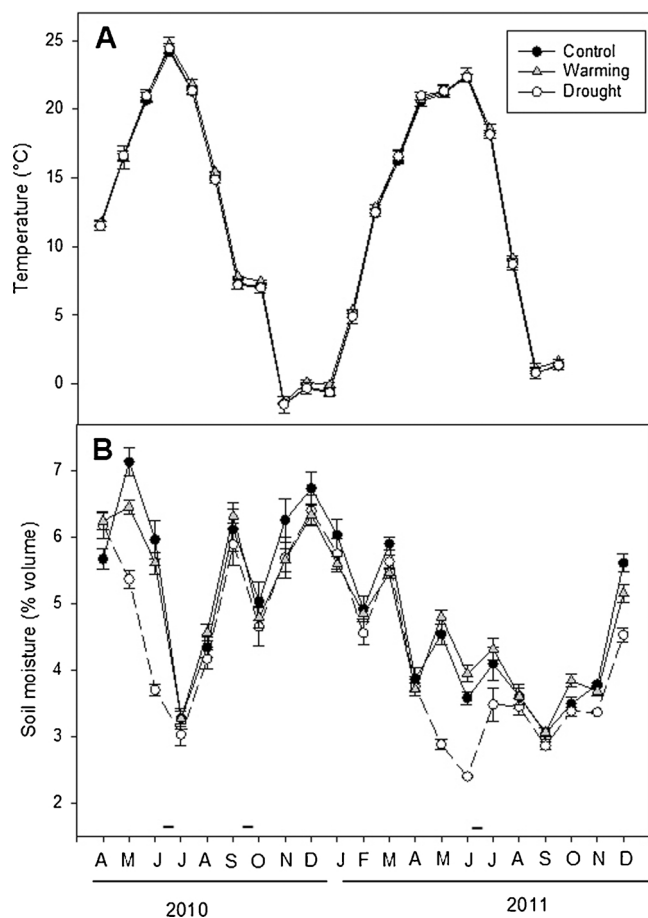


Fig. 1. (A) Monthly air temperature (B) and soil moisture (%volume/volume), through the study period for control (black circles), warming (grey triangles), and drought (white circles) plots. Hyphens indicate campaign periods. Error bars indicate the standard error of the mean ($n = 3$ plots).

Table 2

Warming effects on mean annual air temperature, and drought effects on mean annual soil moisture during the study period (2010–2011).

period	average temperature increase by warming (°C)	Average soil moisture reduction average by drought (%)
2010	0.37 ± 0.06	$11\% \pm 4.6$
2011	0.32 ± 0.03	$17\% \pm 4.7$
2010/2011	0.35 ± 0.03	$13\% \pm 3.2$

74.96% of these spring totals in years 2010 and 2011, respectively. In the long term, the overall decrease in mean annual precipitation was 22% (Kröel-Dulay et al., 2015).

We acknowledge that the temperature increase in warming plots is relatively small, but it roughly equals about three decades of warming observed in the past, and is in the range of temperature increases (0.3–0.7 °C) predicted for the next two decades (IPCC 2014). Also, the annual precipitation decrease of 20–30% is substantial, taking into account that precipitation change scenarios for this region do not predict a major change in annual precipitation.

2.2. Gas exchange measurements

For gas-exchange measurements only mature leaves, positioned on the fifth or the sixth whorl, were selected. Measurements were taken in three different *Populus alba* plants per treatment and season between 10.00 and 16.00 h (solar time). Steady-state net photosynthesis (A),

Table 3

Temperature (°C) and relative humidity (RH) in the leaf chamber during gas exchange sampling.

Parameter	July 2010	September 2010	July 2011
T (°C)	30.70 ± 0.14	27.46 ± 0.56	31.49 ± 0.46
RH (%)	31.37 ± 1.02	31.64 ± 5.13	42.43 ± 0.96

stomatal conductance (g_s) and intercellular CO₂ concentration (C_i) were recorded using an infrared gas analyzer (LI-COR 6400, LI-COR, Lincoln, NE, USA), by enclosing one leaf in a 6 cm² cuvette with a transparent upper Teflon window. Parameters such as relative humidity, air temperature and photosynthetically active radiation (PAR) in the leaf chamber were obtained simultaneously with gas exchange measurements. Measurements were carried out under 400 ppm of CO₂ and a Photosynthetic Photon Flux Density (PPFD) of 1000 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The temperature, and relative humidity in the leaf chamber during the gas exchange sampling are shown in Table 3.

2.3. Isoprene emission

After performing gas exchange measurements, the same leaves were used for measuring isoprene emission (three different *Populus alba* plants per treatment and season). The air leaving the gas-exchange cuvette was passed through a Teflon T connector to a trap (8 cm long and with 0.3 cm internal diameter) filled with 200 mg Tenax particles 35–60 mesh in size (Markes International Limited, Llantrisant, UK). Prior to use, all traps were conditioned for 10 min at 350 °C with a stream of purified helium. A calibrated air sampling pump was used to draw 5 L of the air flowing over the leaf inside the cuvette, through the trap at a rate of 200 mLmin^{−1} during 25 min. VOC background was measured at least three times throughout each day by collecting 5 L of air exiting the empty cuvette in order to take into account background variability. Each sample value was subtracted by the value of the closest blank. After sampling, the traps were stored at 4 °C until analysis (within 24–48 h).

VOCs retained on the adsorption traps were thermally desorbed at 275 °C for 10 min in a Markes Unity 1 thermal desorption unit (Markes International Limited, Llantrisant, UK) under a flow rate of helium, cryofocused in a cold trap containing a 2 mm diameter $\times$ 60 mm long bed of Tenax TA backed up by Carbowgraph 1TDTM separated and supported at each end by quartz wool and kept at −10 °C by a Peltier cell. By rapid heating the cryogenic trap at 300 °C, VOCs were injected into a 30 m MS-5HP capillary column with an inner diameter of 0.25 mm (J&W Scientific USA, Agilent Technologies, Palo Alto, CA, USA), connected to a gas chromatographic-mass spectrometric unit (Agilent GC-MS-MSD 5975C). The column temperature was maintained at 40 °C for 1 min, and then increased up to 210 °C at a rate of 5 °C/min. A final temperature of 250 °C was reached using a rate of 20 °C/min. Helium was used as a carrier gas. The retention time of isoprene is 1.502 min.

The actual concentrations of isoprene in each sample were quantified by reference to an external standard obtained by filling the cartridges with 2 L of air containing 70 p.p.b. of an isoprene gaseous standard (Rivoira, Milan, Italy).

The algorithm of Guenther et al. (1993) was used to normalize isoprene emission rates at 30 °C and 1000 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PFD. We used the algorithm:

$$E = E_s C_T$$

Where C_T is function of temperature:

$$C_T = \exp \{ C_{T1} \cdot (T - T_s) (R \cdot T_s \cdot T)^{-1} \} / 0.961 + \exp \{ C_{T2} \cdot (T - T_m) (R \cdot T_s \cdot T)^{-1} \}$$

Where T was the leaf temperature in degrees Kelvin

R was a gas constant ($8.314 \text{ JK}^{-1} \text{ mol}^{-1}$),

T_s was the normalizing temperature in degrees Kelvin

T_m was an empirical coefficient (314 K),

C_{T1} was an empirical coefficient ($95,000 \text{ J mol}^{-1}$), and

C_{T2} was an empirical coefficient ($230,000 \text{ J mol}^{-1}$)

The percentage of assimilated carbon lost as isoprene (%) was calculated by the following formula:

% assimilated carbon lost as isoprene = $(I \cdot 12) / (A \cdot 60) \cdot 100$

Where I is isoprene emission in $\text{nmol m}^{-2} \text{ s}^{-1}$ and A is in $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$.

2.4. Quantitative mRNA expression levels of *P. alba* isoprene synthase gene

Quantification of isoprene synthase (*PaISPS*) mRNA gene expression was achieved as follows: Leaf samples, from each experimental plot (three different *Populus alba* plants per treatment and season- biological replicates), were collected and used to perform quantitative polymerase chain reaction (qPCR) analyses. For each biological experiment a qPCR analysis was performed as triplicate. One of them was used as a representative picture of our results. Total RNA ($2.5 \mu\text{g}$), extracted from 200 mg fresh mass of leaves at $0\text{--}4^\circ\text{C}$ using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), was reverse-transcribed (RT) with SuperScript II RNaseH reverse transcriptase (Invitrogen, Carlsbad, CA, USA) to synthesize the first-strand cDNA, from $1 \mu\text{g}$ total RNA extracted. Quantitative polymerase chain reactions (qPCRs) were performed by following the protocol described in Fortunati et al., 2008, using an Mx3000P[®] (Stratagene; CA, USA) system. Quantitative PCR reaction was carried out using *P. alba* isoprene synthase gene (*PaISPS*) specific internal primers (sense primer *PaISPS-Fw2*: $5'\text{-GTCGTTTGGAGCATTGAAGCATACCG-}3'$ and antisense primer *PaISPS-Bw2*: $5'\text{-ATGTTTCCCCCTTGTCCTTCAGATTGTGTC-}3'$). *PaISPS* gene expression was normalized using the *ACTIN2* gene, with two specific primers (sense primer *PaACT-Fw1*: $5'\text{-GCAGGCATCCACGAAACCACATACAACCTC-}3'$ and antisense primer *PaACT-Bw1*: $5'\text{-CACCTTGATTTTCATGCTGCTTG GGGC-}3'$). Reactions were performed at the following conditions: 10 min at 95°C , followed by a total of 40 three-temperature cycles (30 s at 95°C , 1 min at 58°C , and 1.5 min at 72°C). Quantification of the qPCR amplification results was performed by constructing cDNA plasmid standards, specific for *PaISPS* and *PaISPS* genes, as described in Fortunati et al., 2008. Using a serial dilution (10 folds) of plasmid cDNA, ranging from 10^{-1} to 10^{-7} input cDNA copies was used as a standard curve (each standard curve was performed as triplicate). All qPCR data were elaborated by the MxPro v3.2 software (Stratagene, CA, USA).

2.5. Statistical analysis

Analyses of variance (ANOVA) with gas exchange, normalized isoprene emission and *PaISPS* gene expression as dependent variables (one mean value per treatment and season; $n = 3$) and treatment and season as independent factors, were conducted using SigmaPlot 12.5 (Systat Software Inc., San Jose, CA, USA). Statistical differences between treatments in each season and between seasons for each treatment were analysed with Tukey post-hoc tests. Different letters mean significant differences in mean values ($P < 0.05$). Linear regressions for normalized isoprene emission and *PaISPS* expression levels were calculated using linear regression analysis function contained in GraphPad Prism software (GraphPad Software Inc., San Diego, Calif.). P values < 0.05 were considered significant.

3. Results

3.1. Gas exchange, isoprene emission and *PaISPS* gene expression

P. alba showed a typical seasonal trend of photosynthetic rates and stomatal conductance with significantly higher average assimilation

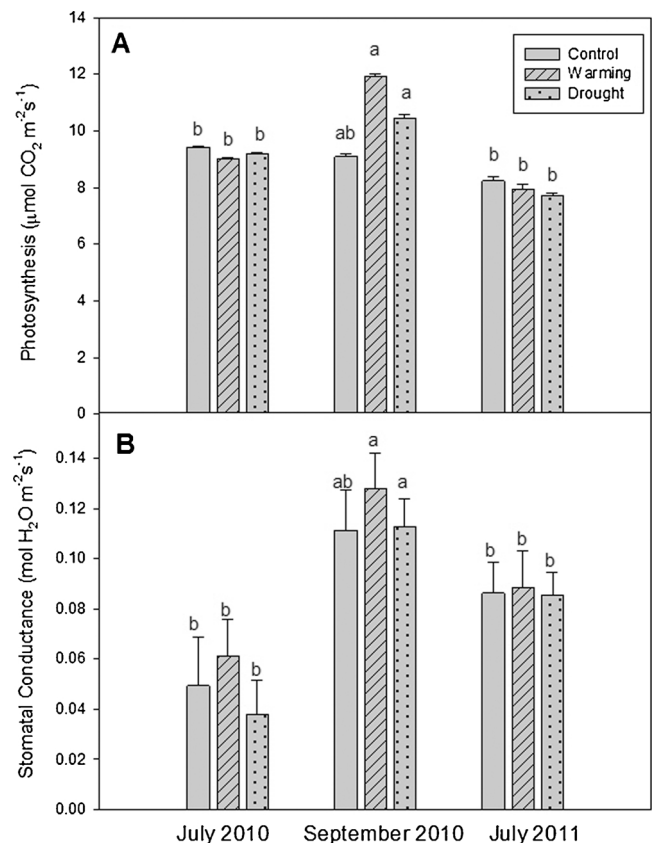


Fig. 2. (A) Net photosynthetic rates ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), (B) stomatal conductance ($\text{mol m}^{-2} \text{ s}^{-1}$) and (B) intercellular CO_2 concentration (ppm), of *P. alba* plants for control (grey full bars), warming (grey lined bars) and drought (grey dotted bars) treatments during summer 2010, autumn 2010 and summer 2011. Mean $\pm$ SE ($n = 3$) is shown. Different letters indicate statistical differences at $P < 0.001$.

($P < 0.001$) values (considering all treatments) in September 2010, ($10.9 \mu\text{mol m}^{-2} \text{ s}^{-1}$) as compared to July 2010 ($9.2 \mu\text{mol m}^{-2} \text{ s}^{-1}$) and July 2011 ($8 \mu\text{mol m}^{-2} \text{ s}^{-1}$) (Fig. 2A). No significant differences were found between treatments (Fig. 2A). Also, g_s changed with the season with lower values during July 2010 ($0.05 \text{ mol m}^{-2} \text{ s}^{-1}$) and 2011 ($0.08 \text{ mol m}^{-2} \text{ s}^{-1}$) than during September 2010 ($0.12 \text{ mol m}^{-2} \text{ s}^{-1}$) ($P < 0.001$) (Fig. 2B). No significant differences were found between treatments (Fig. 2B).

Normalized isoprene emission also showed lower average emission rates (considering all treatments) in July 2010 ($6.28 \text{ nmol m}^{-2} \text{ s}^{-1}$) and July 2011 ($5.94 \text{ nmol m}^{-2} \text{ s}^{-1}$) than in September 2010 ($12.16 \text{ nmol m}^{-2} \text{ s}^{-1}$).

No significant differences were found between treatments in July 2010 and September 2010 (Fig. 3A). However normalized emission rates for drought treated plants were significantly ($p < 0.001$) lower than for control and warming treated plants in July 2011 (Fig. 3A).

PaISPS gene expression showed differences between different periods (Fig. 3A). Average values for all treatments were lower in both summer periods (July 2010, 4.83 and July 2011, 3.87) than in September 2010, (9.01). Moreover, in July 2011, warming treated plants presented lower *PaISPS* gene expression than control and drought treated ones (Fig. 3A).

Average values of percentage of assimilated carbon lost as isoprene (%) (considering all treatments) were statistically higher ($P < 0.01$) in September (0.74%) than in July 2010 (0.35%) and 2011 (0.36%) (Fig. 3B). No significant differences were found between control, warming and drought.

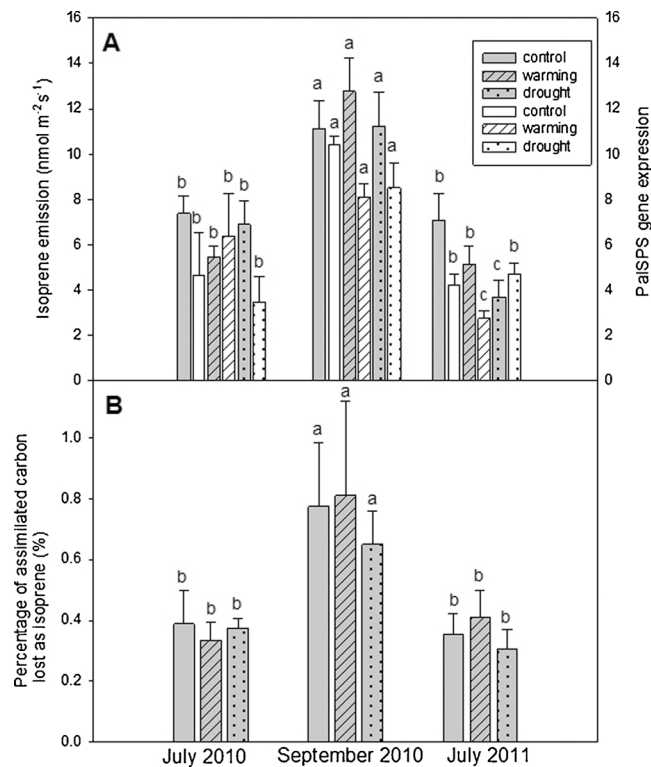


Fig. 3. (A) Normalized isoprene emission rates ($\text{nmol m}^{-2} \text{s}^{-1}$) (grey bars, left axis), and Expression analysis of the isoprene synthase gene in *Populus alba* leaves (*PaISPS*) (white bars, right axis) and (B) % of assimilated carbon lost as isoprene of *P. alba* plants for control (full bars), warming (lined bars) and drought (dotted bars) treatments during summer 2010, autumn 2010 and summer 2011. Mean $\pm$ SE ($n = 3$) is shown. Different letters indicate statistical differences at $P < 0.001$.

3.2. Mechanistic control of isoprene emission

To further investigate the regulation mechanism of isoprene emission under drought and warming treatments, different correlations among isoprene parameters and A, temperature and C_i were represented and analysed.

Whereas isoprene emission was found to be negatively correlated with temperature in the range between 25 °C and 35 °C ($P < 0.001$, $r^2 = 0.7605$) (Fig. 4A), it was positively correlated with A ($P < 0.001$, $r^2 = 0.3262$) (Fig. 4B).

Contrary to isoprene emission, *PaISPS* gene expression changed in positive correlation with temperature when considering summer values ($P < 0.001$, $r^2 = 0.3104$) (Fig. 5).

Correlations between isoprene emission and leaf internal CO_2 were represented for each treatment separately (Fig. 6), and considering only the measurements performed in September 2010 and in July 2011. C_i values in July 2010 were not included in the figure because they differed inconsistently probably due to stomatal regulations longer than usual and likely related to a mild drought stress during midday measurements. Whereas a significant inverse relationship was found between isoprene emission and C_i if comparing control and warming treatments ($P < 0.05$; Fig. 6A and B), a positive correlation between isoprene emission and C_i under drought treatment ($P < 0.001$; Fig. 6C) was observed.

4. Discussion

Neither night-time warming nor increased drought conditions impaired the photosynthetic properties of *Populus alba*, though an inhibition of photosynthesis was expected in plants suffering from water

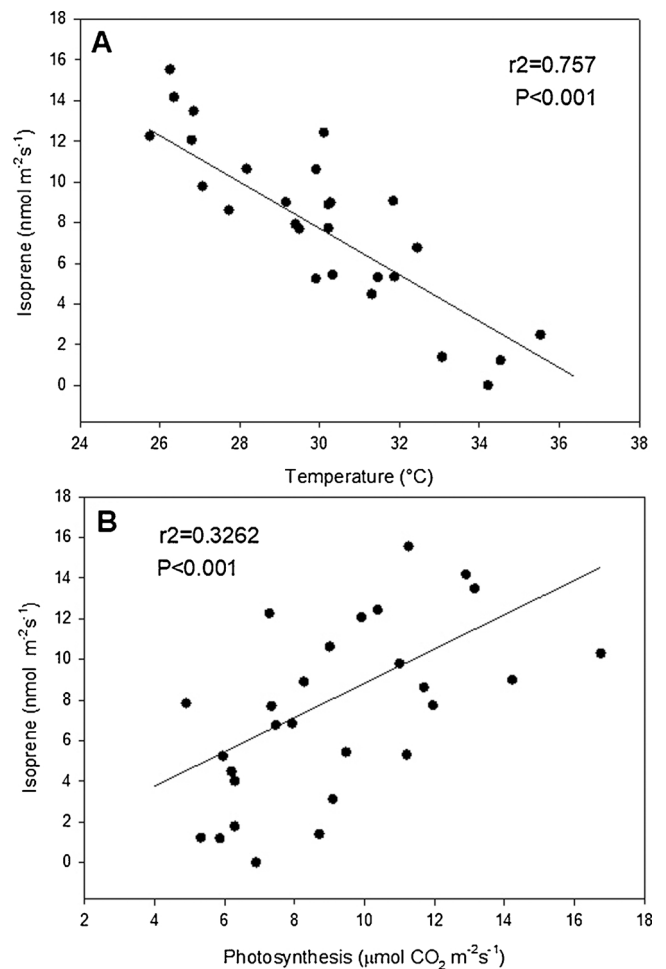


Fig. 4. (A) Correlation between normalized isoprene emission rates ($\text{nmol m}^{-2} \text{s}^{-1}$) and temperature of *P. alba* leaves in control, drought and warming plots. (B) Correlation between isoprene emission rates ($\text{nmol m}^{-2} \text{s}^{-1}$) and net photosynthetic rates ($\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$), of *P. alba* leaves in control, drought and warming plots.

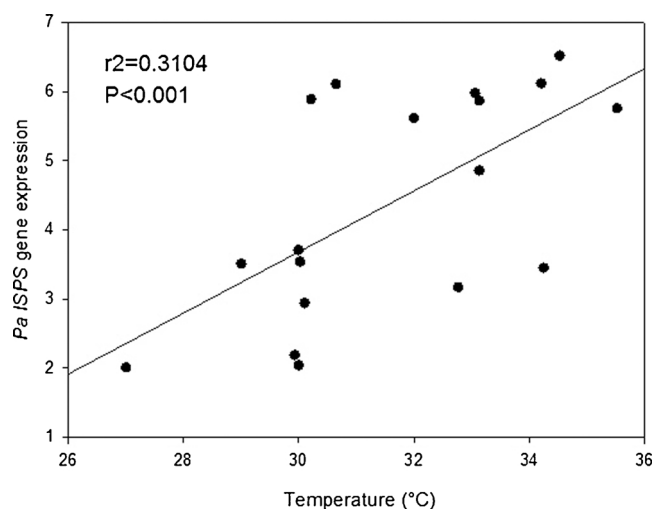


Fig. 5. Correlation between *PaISPS* gene expression and temperature of *P. alba* leaves in control, drought and warming plots during summer measurements.

stress, mainly as a consequence of the increased resistance to CO_2 in both the stomata and the mesophyll (Schulze and Hall, 1982; Ogaya and Penñuelas, 2003; Vitale et al., 2011). Moreover, in *P. alba*, A and g_s

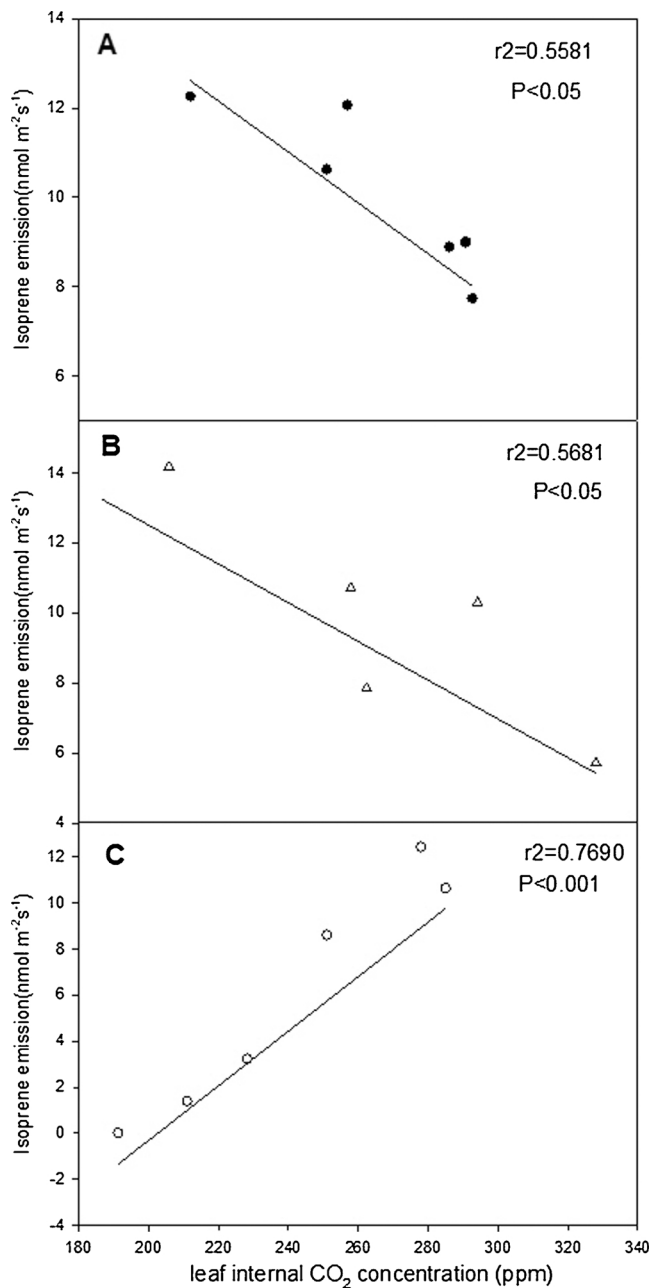


Fig. 6. Correlation between normalized isoprene emission rates ($\text{nmol m}^{-2} \text{s}^{-1}$) and intercellular carbon (ppm) for control *P. alba* leaves (black circles) (A), warming treated leaves (white triangles) (B) and drought treated leaves (white circles) (C) in September 2010 and in July 2011.

are known to decrease under drought stress (Brilli et al., 2007). This was observed during both July campaigns, 2010 and 2011, as a general reduction of photosynthesis in all treatments as compared to the September campaign. In addition, leaf size measurements in the experiment showed that drought treated plants had smaller leaves than control ones (Mojzes et al., 2017), (a typical drought adaptive mechanism to avoid water loss).

The main aim of this study was to document drought and warming effects on *Populus alba* isoprene emission rates. Our results showed a slight drought effect and no warming effect.

P. alba normalized isoprene emission rates ranged between 1.76 and $17.45 \text{ nmol m}^{-2} \text{s}^{-1}$ for control plants, in the range of those reported in most studies for *P. alba*. (Fares et al., 2006; Brilli et al., 2007; Loreto et al., 2007). Temperature is a key factor controlling isoprenoids

emissions from vegetation. However, in our specific case, the average increase of 0.35°C during both years (2010–2011) in night temperature was not enough to increase isoprene emissions during the day. Surprisingly, we found a negative correlation between normalized isoprene emission and temperature when pooling data from all treatments and in the range of temperatures of the leaf chamber during measurements (from 25°C to 35°C degrees) (Fig. 4A).

This result is in disagreement with most studies where the temperature-dependent increase of isoprene emission is exponential, until a maximum emission rate is reached at around 40°C (Loreto and Schnitzler, 2010; Loreto et al., 2006; Fischbach et al., 2000), reflecting the temperature dependence of the specific enzyme, isoprene synthase (Fischbach et al., 2000). However, it is known that longer periods of heat stress can result in a gradual reduction of the emission capacity (Niinemets et al., 2010), likely due to denaturation of biosynthetic enzymes that generally takes place at temperatures ranging between 40°C and 45°C . Thus, the temperature range between 25°C and 35°C seems to be relatively low to induce an inhibition of isoprene emission. Seasonal variation in isoprene emissions may have contributed to this trend, as emissions were higher in September 2010 than in July 2010 and July 2011, despite the lower temperatures in autumn. Several studies already observed that emissions are often lower during summer than during autumn despite the higher temperatures (Bertin and Staudt, 1996; Rivoal et al., 2010). This decrease in isoprene emissions during summer campaigns in July may be well correlated with photosynthesis values, that are higher in autumn than in spring. Indeed, we have found a positive correlation between A and normalized isoprene emission when considering all treatments and dates (Fig. 4B). *PalSPS* gene expression levels could have also contributed to the differences in isoprene emission among seasons, as levels resulted to be higher during the measurements in September than during measurements during both summers (Fig. 3A).

Moreover, *PalSPS* gene expression decreased with warming treatment during July 2011, though *ISPS* gene expression has been reported to increase in response to light and temperature (Sasaki et al., 2005; Cinege et al., 2009) as observed with our data considering only measurements performed during the two campaigns of July (Fig. 5). The fact that isoprene emission and *PalSPS* gene expression changed both with temperature, but in opposite directions (when considering only summer measurements) might be due to a number of co-occurring limiting factors such as water limitation or higher respiration rates which can induce competition for the isoprene precursor DMADP, as observed for instance under elevated CO_2 (Rosenstiel et al., 2003).

Severe drought is known to decrease isoprenoid emission rates (Staudt et al., 2001), but such an inhibition of the emission seems to occur only when the severity of the stress largely inhibits photosynthesis (Staudt et al., 2001; Lavoie et al., 2009). The relationship between BVOC emission and drought over time is clearly shown in a curve by Possell and Loreto (2013). This is explained by the decrease of C_i which ranges between 50 and 80 ppm under mild drought conditions, and can drop to values close to the compensation point under severe drought (Niinemets et al., 2010). Under these conditions, the absence of photosynthetic intermediates may reduce carbon availability for volatile isoprenoid biosynthesis (Niinemets et al., 2010). However, decreases in isoprene emission rarely occur when drought is mild (Sharkey and Loreto, 1993; Llusà and Peñuelas, 1998; Delfine et al., 2005; Brilli et al., 2007). In these cases, an uncoupling between isoprene emission (that remains stable or decreases slightly) and photosynthesis (that decreases dramatically) has been observed for different plant species (Tingey et al., 1981; Sharkey and Loreto, 1993; Fang et al., 1996; Centritto et al., 2011; Dani et al., 2014), including *P. alba* (Brilli et al., 2007). In our case, we observed a reduction of isoprene emissions under drought but only during July 2011. This reduction of isoprene emission seemed to be relatively independent from photosynthesis, indicating a more complex regulation of isoprene emission, likely involving other external (relative humidity, high temperatures) and/or internal factors

(isoprene synthase activity, isoprene synthase protein levels) (Llusia and Peñuelas, 2000). Year 2011 was dryer than year 2010 and moreover, during year 2011 drought treatment was more pronounced causing a 17% reduction in soil moisture compared to control. The mild long-term drought exposure in combination with a higher summer drought stress in year 2011 and with summer high temperatures might have altered the processes of isoprene formation and emission. Fortunati et al., 2008 observed that in plants grown at high temperatures and characterized by high isoprene emission rates, specific activity of ISPS decreased during the early stages of drought stress.

We observed a significant inverse relationship between isoprene emission and Ci if comparing control and warming treatments (Fig. 6A and B). This relationship possibly reflects the competition for PEP substrate between PEP carboxylase, whose activity likely strongly correlates with CO₂ concentration, and isoprene emission (Rosenstiel et al., 2003, 2006). An inverse relationship between isoprene emission and Ci has been also observed in hybrid aspen (Rasulov et al., 2009) and poplar genotypes of different species (Guidolotti et al., 2011).

On the other hand, we observed a positive correlation between isoprene emission and Ci under drought treatment (Fig. 6C), suggesting a change in the regulation of pyruvate feeding into the DOXP/MEP pathway, as has been previously shown for black poplar under drought stress (Fortunati et al., 2008). Fortunati et al., 2008 reported a positive relationship between isoprene emission and PEP carboxylase activity in poplar leaves grown at 25 °C (but not at 35 °C) under drought stress. The fact that isoprene emission was lower at 25 °C than at 35 °C (due to the temperature dependency of isoprene synthase activity), could have allowed PEP concentration to support both, respiration and isoprene biosynthesis during drought stress.

Alternatively, under drought stress isoprene synthesis could be partially supported by alternative carbon sources such as old or temporary stored photosynthates. It is already known that approximately 70–90% of the carbon used for isoprene synthesis originates directly from the photosynthetic carbon flux, and that the remaining carbon is derived from alternative sources (Sanadze et al., 1972; Affek and Yakir, 2003; Schnitzler et al., 2004).

In this study, the percentage of assimilated carbon lost as isoprene was in the range between 0.3–and 1%, which is in agreement with what was reported in other studies (Sharkey and Yeh, 2001; Guidolotti et al., 2011). It is interesting to note that neither warming nor drought showed any significant effect on this, while the difference between July (of both years) and September was quite evident, mainly driven by emission values. This is somehow surprising because under growing stress conditions, often assimilation rates decrease dramatically or at higher rates than isoprene emissions (Medori et al., 2012; Nogués et al., 2015) inducing an increase of the ratio between carbon lost over carbon assimilated.

In conclusion, our study showed that isoprene emission and isoprene synthase expression levels were affected by open-field drought and warming, respectively but only in a single campaign. This suggests that treatments, even if carried out over long-term, were likely not severe enough to induce a permanent adaptive response on isoprene parameters. A clear response was driven by seasonality for both, isoprene emission and *PaISPS* expression levels, that were lower in summer than in autumn, similarly to what observed for photosynthesis. Moreover, when considering summer measurements, a positive relationship was found between *PaISPS* expression levels and temperature, which is the opposite of what found for isoprene emission. This suggests the presence of limiting factors on substrate availability such as water scarcity or increasing respiration rates, which could induce a competition for DMADP. Finally, our study showed a positive relationship between isoprene emission and Ci under drought, but not under control or warming, suggesting an increased PEP availability for DMADP formation and isoprene synthesis under drought conditions likely due to either a decreased consumption of PEP for the mitochondrial respiration or to the existence of alternative carbon sources.

Acknowledgements

The authors thank the financial support by the European Union FP7-Infrastructure-2008-1 project INCREASE, contract no. 227628. The research was also supported by NPU I programme (LO1415), the Ministry of Education, CZ (LD13031), the Hungarian Scientific Research Fund (OTKA K112576, PD115637), and the MTA János Bolyai Research Scholarship. The publication was financially supported by the Ministry of Education and Science of the Russian Federation (the Agreement №02.A03.21.0008).

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